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Sent: Monday, August 18, 2003 5:03 PM
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TI Inhibition of NF-AT signal transduction events by a dominant-negative form of calcineurin
AU Muramatsu, Taro; Kincaid, Randall L.
SO Biochemical and Biophysical Research Communications (1996), 218(2), 466-72

TI Regulation of AP-1 and NFAT transcription factors during thymic selection of T cells.
AU Rincon M; Flavell R A
SO MOLECULAR AND CELLULAR BIOLOGY, (1996 Mar) 16 (3) 1074-84.

TI Transcription mediated by NFAT is highly inducible in effector CD4+ T helper 2 (Th2) cells but not in Th1 cells.
AU Rincon M; Flavell R A
SO MOLECULAR AND CELLULAR BIOLOGY, (1997 Mar) 17 (3) 1522-34.

TI Role of NFATx (NFAT4/NFATc3) in expression of immunoregulatory genes in murine peripheral CD4+ T cells.
AU Chen Jingtao; Amasaki Yoshiharu; Kamogawa Yumiko; Nagoya Miho; Arai Naoko;
SO JOURNAL OF IMMUNOLOGY, (2003 Mar 15) 170 (6) 3109-17.

TI Requirement for transcription factor NFAT in interleukin-2 expression.
AU Chow C W; Rincon M; Davis R J
SO MOLECULAR AND CELLULAR BIOLOGY, (1999 Mar) 19 (3) 2300-7.

TI Nf1-regulated adenylyl cyclase pathway.
AU Tong, J. (1); Guo, H.; Hannan, F.; Hakker, I.; An, J.; Zhong, Y.
SO Society for Neuroscience Abstracts, (2000) Vol. 26, No. 1-2, pp. Abstract No.-345.9. print.
Meeting Info.: 30th Annual Meeting of the Society of Neuroscience New Orleans, LA, USA November 04-09, 2000 Society for Neuroscience . ISSN: 0190-5295.

TI All-trans retinoic acid corrects GM-CSF hypersensitivity in Nf-1 -/- knock-out hematopoietic progenitor cells in vitro.
AU Robertson, K. A. (1); Zhang, Y. (1); Clapp, D. W. (1)

Role of NFATx (NFAT4/NFATc3) in Expression of Immunoregulatory Genes in Murine Peripheral CD4$^+$ T Cells[1]

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Ca^{2+}-regulated NFAT family members are transcription factors crucial for the expression of various cytokine genes and other immunoregulatory genes. Analyses of mice defective in one or two NFAT family members have revealed functions specific to each *NFAT* gene. However, the redundant functions of several family members limit the usefulness of gene disruption analysis. For example, CD4$^+$ T cells isolated from NFATx-disrupted mice do not show any modulation in cytokine gene expression, perhaps because other family members compensate for its absence. To analyze the role of NFATx in the regulation of immunoregulatory genes in T cells, we made a gain-of-function mutant by creating transgenic mice expressing a constitutively nuclear form of NFATx in T cell lineages. In naive CD4$^+$ T cells, NFATx up-regulated the expression of several cytokine genes and activation markers and suppressed the expression of CD154. In Th1 cells, NFATx enhanced the expression of the Th1 cytokine genes, *IFN-γ* and *TNF-α*. In contrast, NFATx suppressed Th2 cytokine genes such as *IL-4* and *IL-5* in Th2 cells. It has been reported that both NFAT1 and NFATx are required to maintain the homeostasis of the immune system. Our results suggest that NFATx exerts this function by inhibiting the expression of some critical immunoregulatory genes. *The Journal of Immunology,* 2003, 170: 3109–3117.

Cytokine gene expression in T cells is induced by the engagement of the TCR and/or the activation of costimulatory receptors such as CD28. However, patterns of cytokine gene expression vary according to the lineage of the cell receiving the activation signal. When a naive CD4$^+$ T cell released from the thymus encounters an Ag presented in the correct context by dendritic cells or macrophages, it begins to produce IL-2, a potent T cell growth factor, and to differentiate into one of two Th subsets, Th1 and Th2. Th1 cells produce inflammatory cytokines, such as IFN-γ and TNF-α, and are responsible for the eradication of intracellular pathogens. Th2 cells produce IL-4, IL-5, IL-10, IL-13, and others, some of which protect against extracellular pathogens such as nematodes. In addition, some cytokines, such as IL-3 and GM-CSF, are produced by both subsets.

Ca^{2+}-regulated NFAT family proteins were initially identified as transcriptional activators for the stimulation signal-dependent induction of various cytokine genes, such as *IL-2*, *GM-CSF*, and *IL-4*, in T cells (1–4). However, Ca^{2+}-regulated *NFAT* family genes are expressed in precursors as well as in differentiated effectors of Th1 and Th2, hence their distribution could not explain the different patterns of cytokine gene expression in cells of different lineages.

Recently, mice with single and double knockouts (KO)[4] of *NFAT* family genes have been produced, and their functions have been assessed in vivo. Chimeras with recombination-activating gene-deficient mice were produced to analyze phenotypes of lymphocytes and immune responses when the NFAT deficiency was embryonic lethal. Analyses of the T cells derived from these genetically modified mice have revealed distinct functions for each NFAT family member, including various specificities in cytokine gene induction and immunosuppressive activities to maintain homeostasis of the immune system. However, it has become clear that these loss-of-function mutants cannot be used to identify overlapping functions among Ca^{2+}-regulated NFAT family members. For example, NFATc-deficient T cells derived from NFATc KO chimeric mice are impaired in their ability to express IL-4, but not IL-2 (5, 6), and T cells derived from double NFATc/NFAT1 KO mice are severely impaired in their ability to produce both cytokines (7). These data suggest that either NFATc or NFAT1 can induce IL-2, but that the expression of IL-4 depends more on NFATc. The phenotype of CD4$^+$ T cells from NFAT1-deficient mice is complicated. The induced level of IL-4 mRNA is lower, but the expression is maintained for a longer time, and the overall production of IL-4 seems to be higher in NFAT1-deficient T cells (8–13). Therefore, NFAT1 is critical to both inducing and modulating the amount of IL-4 in vivo. In contrast, IFN-γ production is partially impaired in NFAT1-deficient but not NFATc-deficient T cells (6, 14). When both genes are disrupted, the expression of IFN-γ is severely impaired, indicating that, although NFAT1 is more critical than NFATc in the expression of IFN-γ, both genes are important (7). Peripheral CD4$^+$ T cells of NFATx-deficient mice do not show any effects on cytokine expression patterns (15). Interestingly, the double NFAT1/NFATx KO mice suffer from

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Received for publication June 13, 2002. Accepted for publication January 13, 2003.

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[1] This work was supported by grants from Japan Health Science Foundation and Japan Science and Technology Cooperation.

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[4] Abbreviations used in this paper: KO, knockout; cnNFATx, constitutively nuclear NFATx; CRI, calcineurin-regulated inhibitory sequence; SP, serine proline box/repeat; CD62L, CD62 ligand; CD40L, CD40 ligand; CsA, cyclosporin A; IRES, internal ribosome entry site; EGFP, enhanced green fluorescent protein; CD95L, CD95 ligand.

0022-1767/03/$02.00

lymphoproliferative disorders, including allergic blepharitis, interstitial pneumonia, and lymphadenopathy (16). Significant numbers of peripheral T cells in these mice are activated and produce various cytokines without immunization, and the pattern of cytokine production is skewed toward a Th2 response. Furthermore, naive $CD4^+$ T cells isolated from the double NFAT1/NFATx KO mice showed CD28-independent activation of proliferation and more rapid progression through the cell cycle (17). These analyses suggest that NFATx plays a role in the regulation of T cell functions and immune responses despite the absence of any alterations in T cell function in NFATx-deficient $CD4^+$ T cells.

To gain insights into the differential function of NFATx, we decided to create a gain-of-function mutant by creating transgenic mice expressing a constitutively nuclear form of NFATx (cnNFATx), Δ(CRI · SP12), in T cell lineage (18, 19). The cnNFATx mutant Δ(CRI · SP12) has a deletion in the critical regions of the Ca^{2+} regulatory domain. These regions are the calcineurin-regulated inhibitory sequence (CRI)/serine-rich region, serine proline box/repeat (SP)1, and SP2. The exogenously expressed mutant localizes mainly in the nucleus in the absence of a Ca^{2+} signal. The transcriptional activity of this mutant measured by the reporter plasmid carrying NFAT/AP-1 composite sites in the presence of PMA is comparable to or higher than that of the wild-type NFATx stimulated by PMA and Ca ionophore. Thus, in addition to the effects of overexpression, the activity of this mutant can be distinguished from that of the endogenous wild-type NFATx by comparing their responses under two different conditions: stimulation solely by PMA and stimulation by PMA and Ca ionophore.

In this study, we report that NFATx functions as a positive regulator for *IL-2* and Th1 cytokine genes such as *IFN-γ* and *TNF-α*. In addition, NFATx is able to enhance the expression profile of cell surface activation markers such as CD25 (the IL-2R α-chain), CD69, and CD62 ligand (CD62L). In contrast, NFATx suppressed Th2 cytokine genes, such as *IL-4* and *IL-5*, and an important immunoregulatory molecule, CD154 (CD40 ligand (CD40L)), required for T cells and APC to coordinate the immune response. These data further support the idea that NFATx has some activator functions that can compensate for the absence of other NFAT family members. However, more important is the finding that NFAT1 and NFATx cooperate with each other to suppress T cell functions by affecting the expression of critical immunoregulatory molecules and modulating cell proliferation to maintain the homeostasis of the immune system.

Materials and Methods

Mice

The cnNFATx Δ(CRI · SP12) was cloned into a plasmid carrying the *lck* proximal promoter and a portion of the human growth hormone gene in which the initiation codon was mutated (Fig. 1) (18, 20). In the resulting plasmid, the cnNFATx cDNA is flanked by the mouse *lck* proximal promoter at the 5′ end and the portion of the human growth hormone gene at the 3′ end. The *Not*I fragment of the plasmid DNA was purified and injected into C57BL/6 embryos. Two lines of mice transgenic for cnNFATx in a C57BL/6 background and expressing the transgene in peripheral T cells were used at ages 5–15 wk for experiments. Mice were screened for the presence of the transgene by PCR amplification of tail DNA. Primers used were 5′-GTGTGATGTCTCCCAGGTAGTCC-3′ and 5′-GGGCGC CACAGTTTGCAGTAGTCATC-3′. DO11.10 αβ TCR-transgenic mice were backcrossed with cnNFATx-transgenic mice. F_1 mice were used to prepare polarized Th1 and Th2 populations. All the experiments were performed on mice from two lines of founder mice.

Cell purification and culture

T cells were maintained in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, 50 μM 2-ME, 1 mM sodium pyruvate (all from Life Technologies, Gaithersburg, MD) and appropriate cytokines. T cell suspensions were prepared from lymph node and spleen cells. $CD4^+$ T cells were purified using magnetic beads (Dynabeads L3T4; Dynal, Lake Success, NY). For primary stimulation, purified $CD4^+$ T cells were stimulated in vitro with 10 ng/ml PMA (Nacalai Tesque, Kyoto, Japan), 0.2 μg/ml ionomycin (Nacalai Tesque), and 1.0 μg/ml cyclosporin A (CsA; Calbiochem-Novabiochem, La Jolla, CA) at 37°C in a humidified atmosphere with 5% CO_2. For selected experiments, $CD4^+$ T cells were further purified into a $CD44^{low}$ $CD4^+$ population by cell sorting. Culture conditions for polarized Th1 and Th2 populations derived from DO11.10 αβ TCR-transgenic mice have been previously described (21). All experiments were performed in parallel using equal numbers of cells from cnNFATx-transgenic mice and wild-type littermates.

Preparation of nuclear extract

Nuclear extracts from $CD4^+$ T cells were prepared as described, with some modification (22). In brief, after stimulation, $CD4^+$ T cells were washed twice with ice-cold PBS, resuspended in buffer A (10 mM HEPES (pH 7.6), 0.1 mM EDTA, 0.1 mM KCl, and 2 mM $MgCl_2$), and incubated on ice for 5 min. An equal amount of buffer B (buffer A plus 0.2% Nonidet P-40) was added and mixed, and the resulting nuclei were pelleted by brief, low-speed centrifugation. The nuclear pellet was washed with buffer A, resuspended in 200 μl of buffer C (50 mM HEPES (pH 7.9), 50 mM EDTA, 50 mM KCl, and 10% glycerol) containing 0.3 M ammonium sulfate (pH 7.9), and vortexed vigorously for 30 min at 4°C. The debris was pelleted by high-speed centrifugation at 70,000 rpm for 45 min, and the proteins in the supernatant were precipitated by adding an equal volume of 3.0 M ammonium sulfate (pH 7.9). The suspension was incubated on ice over night and centrifuged at 50,000 rpm for 15 min. The nuclear proteins

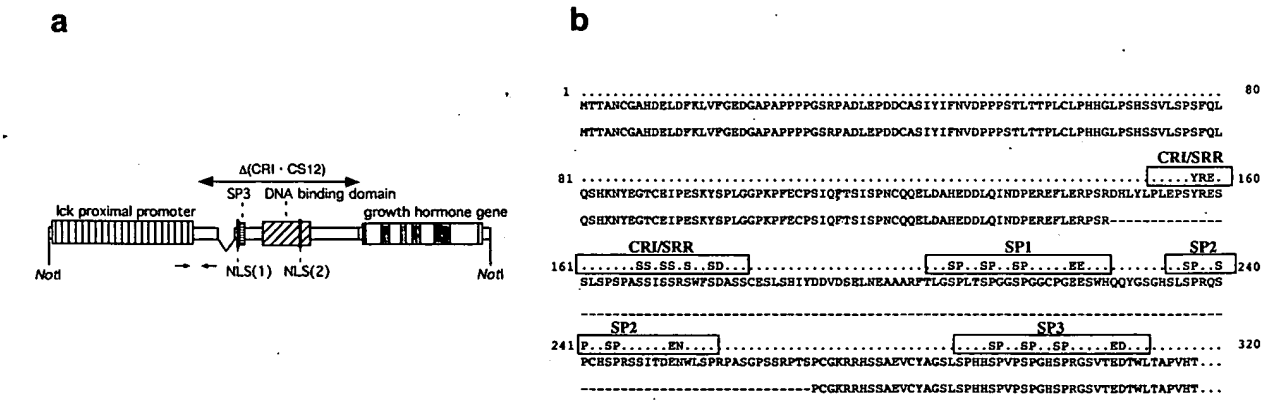

FIGURE 1. Structure of the construct used to generate cnNFATx-transgenic lines. *a*, Schematic diagram of the construct. The region derived from cnNFATx mutant is indicated by a long arrow. Short arrows represent primers used for the screening. Gray boxes indicate exons of the human growth hormone gene. *b*, An alignment of the deleted amino acid residues of NFATx. Important motifs in the Ca^{2+} regulatory domain are indicated. The region encompassing from D147 to S269 is deleted. SRR, Serine-rich region.

in the pellet were dissolved in 20 μl of buffer C and stored at −80°C until use. All buffers were supplemented with 1 mM DTT, 1 mM PMSF, and 2 mg/ml leupeptin (all from Sigma-Aldrich, St. Louis, MO). Protein concentration was estimated with a BCA protein assay kit (Pierce, Rockford, IL).

Antibodies

Anti-mouse NFATx polyclonal Ab, DN97, was prepared as described (19). Anti-mouse NFAT1 mAb and anti-mouse NFATc mAb were purchased from Affinity BioReagents (Golden, CO) and Upstate Biotechnology (Lake Placid, NY), respectively. Anti-c-Jun polyclonal Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

EMSA

NFAT-DNA reactions were conducted with 2 μg of nuclear extracts in a 15-μl solution containing 20 mM HEPES (pH 7.9), 2 mM EDTA, 2 mM DTT, 10% glycerol, 0.1% Nonidet P-40, 3.75 μg of BSA, and 0.375 μg of poly(deoxyinosinic-deoxycytidylic) acid. The reactions were incubated for 20 min at room temperature with a radiolabeled double-stranded oligonucleotide probe. The probe used in these studies contained the NFAT/AP-1 composite site from the IL-2 promoter: 5′-CAAAGAGGAAAATTTGTT TCATACAGAAGGCGTTCA-3′. The samples were separated on 4% polyacrylamide gels. Cold oligonucleotide competitors were added to the reaction mixture when indicated. The AP-1 and SP1 consensus sequence oligonucleotides were used as competitors: AP-1, 5′-GGCGCTTGAT GAGTCAGCCGGAA-3′; and SP1, 5′-GGATTCGATCGGGGCGGGGC GAGC-3′. For supershift assays, anti-NFAT or anti-c-Jun Abs were added to the reaction mixture and incubated for an additional 20 min at room temperature.

Proliferation assay

Purified $CD4^+$ T cells (1×10^5/well) were plated in triplicate onto 96-well round-bottom plates and activated with PMA, PMA plus ionomycin, or PMA plus ionomycin plus CsA for 48 h before harvest. Nonactivated cells were used as controls. [^{3}H]Thymidine (1 μCi/well) was added for the last 8 h. The incorporation of [^{3}H]thymidine was measured with a MicroBeta (PerkinElmer Life Sciences, Boston, MA).

Flow cytometric analysis

FITC-labeled anti-CD4 mAb as well as the PE-labeled mAbs anti-CD25, anti-CD44, anti-CD62L, anti-CD69, and anti-CD154 were purchased from BD PharMingen (San Diego, CA). Expression of cell surface molecules on $CD4^+$ T cells was analyzed by flow cytometry 6 h (CD154) and 24 h (CD25, CD44, CD62L, and CD69) after stimulation. Single-cell suspensions of spleen cells were incubated with mAbs at 4°C and analyzed on a FACScan flow cytometer with CellQuest software (BD Biosciences, San Jose, CA). For intracellular staining, Th1 and Th2 cells differentiated for 2 wk were stimulated with PMA and/or ionomycin for 6 h. In the last 2 h, monensin (2 μM) was added, and cells were collected and fixed with 4% formaldehyde for 10 min at room temperature. Fixed cells were washed with PBS containing 0.1% BSA. After 20-min incubation in permeabilization buffer (PBS containing 0.5% saponin (Calbiochem-Novabiochem)) at room temperature, cells were stained for 30 min with PE-conjugated or FITC-conjugated cytokine IFN-γ and IL-4 Abs at 4°C, washed twice with permeabilization buffer, and resuspended in PBS plus 1% FCS. Cell fluorescence was measured using a FACScan flow cytometer, and data were analyzed by CellQuest software. Each experiment was repeated a minimum of three times with similar results.

ELISA

Cell culture supernatants were collected 24 h after stimulation, and cytokine levels for IL-2, IL-4, TNF-α, IFN-γ, IL-5, and IL-10 were analyzed using ELISA kits from BD PharMingen, and IL-13 levels were analyzed with a kit from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. Calculated values are expressed as mean ± SEM. Each experiment was repeated a minimum of three times with similar results.

Real-time quantitative RT-PCR analysis

$CD4^+$ T cells (1×10^7) were either not stimulated or stimulated by PMA alone, PMA plus ionomycin, or PMA plus ionomycin plus CsA for 4 h, and total RNA was extracted by TRIzol method (Invitrogen, Carlsbad, CA). cDNA was generated from 2 μg of total RNA with random hexamers and the Superscript II RT System (Invitrogen). For real-time quantitative RT-PCR, experiments were performed with FastStart DNA Master SYBR green I, LightCycler instrument and relative quantification software (all from Roche Diagnostics, Indianapolis, IN). All results were normalized with respect to the expression of β-actin. The sequences of the primers are as follows: β-actin, 5′-CCTGTATGCCTCTGGTCGTA-3′ and 5′-CCAT CTCCTGCTCGAAGTCT-3′; Egr2, 5′-GGATGTGTTGTGGTGGTCTT-3′ and 5′-GCGGTCATCATTTGCTCCTC-3′; Egr3, 5′-AGCGACTCGG TAGCCCATTA-3′ and 5′-GTAGGTCACGGTCTTGTTGC-3′; T-bet, 5′-TGCCTGCAGTGCTTCTAACA-3′ and 5′-TGCCCCGCTTCCTCTC CAACCAA-3′; and GATA-3′, 5′-AGAACCGGCCCCTTATCAA-3′ and 5′-AGTTCGCGCAGGATCTCC-3′. LightCycler PCR comprised a 10-min denaturation at 95°C, followed by cycles of 95°C for 15 s, 56°C for 10 s, 72°C for 5 s, and cooling on 40°C for 30 s. Each experiment was repeated a minimum of three times with similar results.

Retrovirus vectors and infection

Purification and primary stimulation of naive T cells from DO11.10 αβ TCR-transgenic mice was performed as previously described (21). The bicistronic retrovirus plasmid pMX contains internal ribosome entry site (IRES) and enhanced green fluorescent protein (EGFP). Retrovirus vectors pMX-IRES-EGFP and the Phoenix-Eco packaging cell line were described previously (23, 24). The retrovirus vector encoding the cnNFATx Δ(CRI · SP12) was made by introducing the *Eco*RI-*Xho*I fragment of pMEmX-Δ(CRI · SP12) into the pMX-IRES-EGFP (18). The pMX-cnNFATx-IRES-EGFP construct was confirmed by DNA sequencing. Retroviral transduction of T cells was conducted as previously described with some modification (24, 25). One day after primary antigenic stimulation under Th1/2-polarizing conditions, cells were infected with retrovirus-containing supernatants in the presence of 0.5 μg/ml polybrene (Sigma-Aldrich). Cells were infected with pMX-EGFP or pMX-cnNFATx-IRES-EGFP. On day 7, cells were sorted on the basis of EGFP expression, stimulated again, and cultured for another week under the polarizing conditions. Cytokine production was analyzed by intracellular staining. This experiment was repeated twice.

Results

Expression of the cnNFATx in peripheral T cells

Linearized plasmid DNA carrying cnNFATx driven by the *lck* proximal promoter was injected into fertilized eggs to create transgenic mice in a C57BLACK/6 background (Fig. 1) (20). Although the *lck* proximal promoter is reported to be specific to thymic T cells, RT-PCR detected the expression of the transgene in peripheral $CD4^+$ T cells from some lines. Two lines of these mice were used for all experiments. We confirmed the expression of the cnNFATx protein in these cells with EMSA using nuclear extracts of the resting $CD4^+$ T cells from the spleens and the lymph nodes and a radioactive probe harboring the composite NFAT-AP1 site from the mouse *IL-2* gene. As shown in Fig. 2*a*, NFAT DNA binding activity was much more intense in resting $CD4^+$ T cells isolated from cnNFATx-transgenic mice than in the same cells from wild-type littermates. A supershift assay with Abs against NFAT1, NFATc, and NFATx showed that, while the NFAT DNA binding activity in the resting $CD4^+$ T cells from wild-type littermates was due mostly to NFAT1, the activity in the cnNFATx-transgenic mouse is the result of the accumulation of NFATx (Fig. 2*b*). This finding indicates that the cnNFATx is expressed and localized in nucleus in the absence of a Ca^{2+} signal. Treatment of the resting $CD4^+$ T cells with PMA induced a slowly migrating complex. A supershift assay with Abs against various NFAT family members as well as c-Jun revealed that the complex contained both AP-1 and NFAT. Nuclear extracts from PMA-treated $CD4^+$ T cells from the cnNFATx-transgenic mice contained a large amount of a complex containing AP-1 and NFATx, indicating that cnNFATx is fully activated by PMA in the absence of a Ca^{2+} signal. Although CsA treatment reduced the amount of NFAT complex even in the nuclear extract of the cnNFATx-transgenic T cells, a still significant amount of NFAT activity was retained in the nucleus, suggesting resistance against the effect of CsA. cnNFATx mutant still retains SP3 motif harboring serine residues to be phosphorylated by the various NFAT kinases. This might be the reason for this reduction.

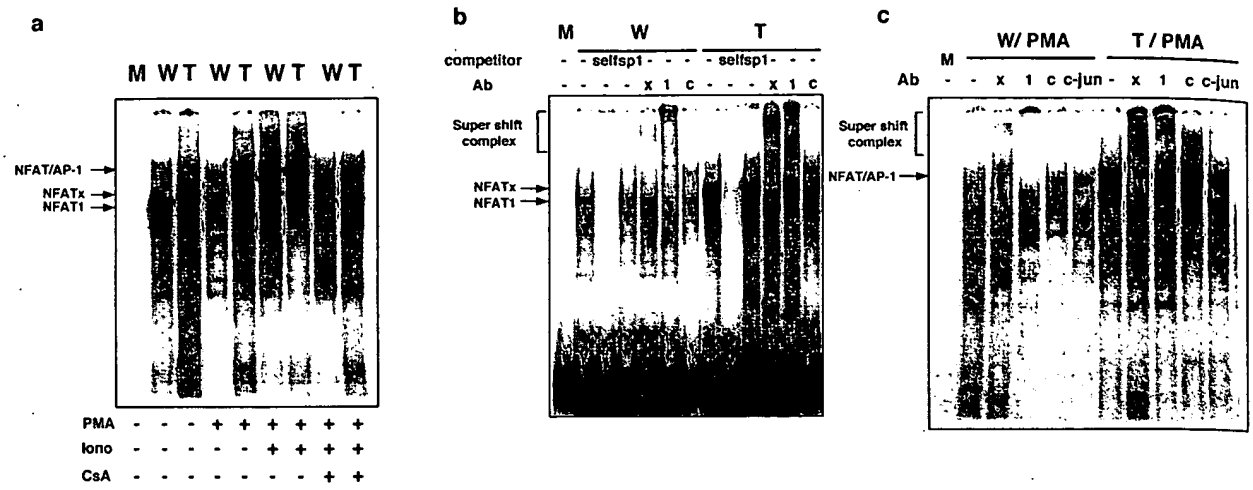

FIGURE 2. Nuclear localization of the cnNFATx protein in peripheral CD4+ T cells derived from the cnNFATx-transgenic mice. *a*, EMSA was conducted with the ^{32}P-labeled oligonucleotide harboring the NFAT/AP-1 composite site derived from the mouse IL-2 promoter and 2 μg of the nuclear extracts prepared from the CD4+ T cells of cnNFATx-transgenic mice (T) or wild-type littermates (W) stimulated for 3 h under various conditions. The mock reaction (M) contained no nuclear extract. *b*, EMSA was conducted with nuclear extracts prepared from the resting CD4+ T cells of cnNFATx-transgenic mice (T) and wild-type littermates (W). To confirm the sequence specificity of the shifted protein-DNA complex, the same nonradioactive oligonucleotide was used as a self-competitor. An oligonucleotide carrying the SP1 binding site was used as a nonspecific competitor. Competitor DNA was added at 100-fold excess over the probe DNA. Each NFAT family member-specific Ab, anti-mouse NFATx Ab (x), anti-mouse NFAT1 mAb (1), and anti-mouse NFATc mAb (c), was added before incubation with the ^{32}P-labeled NFAT/AP-1 probe. *c*, EMSA was performed with nuclear extracts derived from CD4+ T cells of cnNFATx-transgenic mice (T) and wild-type littermates (W) after stimulation solely with PMA for 3 h. Ab against each NFAT family member or anti-mouse c-Jun Ab was used for supershift analysis.

Proliferation and IL-2 expression of transgenic CD4+ T cells are up-regulated

To analyze the effect of cnNFATx on the proliferation of CD4+ T cells, isolated T cells were stimulated with PMA, PMA plus ionomycin, or PMA plus ionomycin plus CsA. As shown in Fig. 3*a*, CD4+ T cells isolated from the cnNFATx-transgenic mice proliferated when treated with PMA alone while those from the wild-type littermates did not. Furthermore, CsA completely suppressed the proliferation of the wild-type littermate T cells stimulated by PMA plus ionomycin, but the T cells from the cnNFATx-transgenic mice showed significant resistance to this effect. This indicates that NFATx facilitates the proliferation of stimulated CD4+ T cells. Because the proliferation of CD4+ T cells in vitro has been shown to depend partly on the production of the T cell growth-promoting cytokine IL-2, we also analyzed the expression of both IL-2 and CD25, which is critical for the expression of the high affinity IL-2R on T cells. As shown in Fig. 3*b*, CD4+ T cells from the transgenic mice produced IL-2 in the presence of PMA alone, although the level of production was relatively low. As shown in Fig. 4, CD25 was partially induced upon PMA treatment in wild-type CD4+ T cells, but CD25 levels were significantly higher in transgenic cells treated with PMA. Thus, the up-regulation of both IL-2 production and CD25 expression presumably synergize to stimulate proliferation in the presence of PMA alone. This supposition is further confirmed by the suppression of proliferation when Abs against each chain of the IL-2R complex were added (data not shown).

The expression of IFN-γ was detected with PMA treatment alone; however, this level of IFN-γ production was quite low compared with the level induced by the full activation, and CsA completely suppressed the expression (Fig. 3*b*). These indicate that the

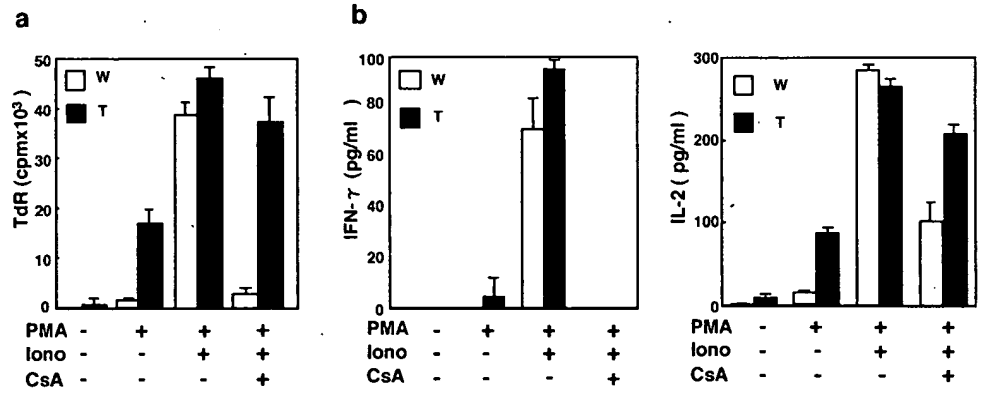

FIGURE 3. Proliferation and cytokine production of cnNFATx naive CD4+ T cells. *a*, Proliferation of CD4+ T cells derived from cnNFATx-transgenic mice (T, ■) or wild-type littermates (W, □) stimulated with PMA, PMA plus ionomycin, or PMA plus ionomycin plus CsA was measured by [^{3}H]thymidine incorporation. Cells (1×10^5) were stimulated for 48 h before harvest. [^{3}H]Thymidine was added in the last 8 h. Proliferation of the transgenic T cells was induced by the stimulation with PMA alone. *b*, ELISA was used to measure IL-2 and IFN-γ levels in the supernatant of the CD4+ T cells derived from the cnNFATx-transgenic mice or wild-type littermates stimulated for 24 h with PMA, PMA plus ionomycin, or PMA plus ionomycin plus CsA.

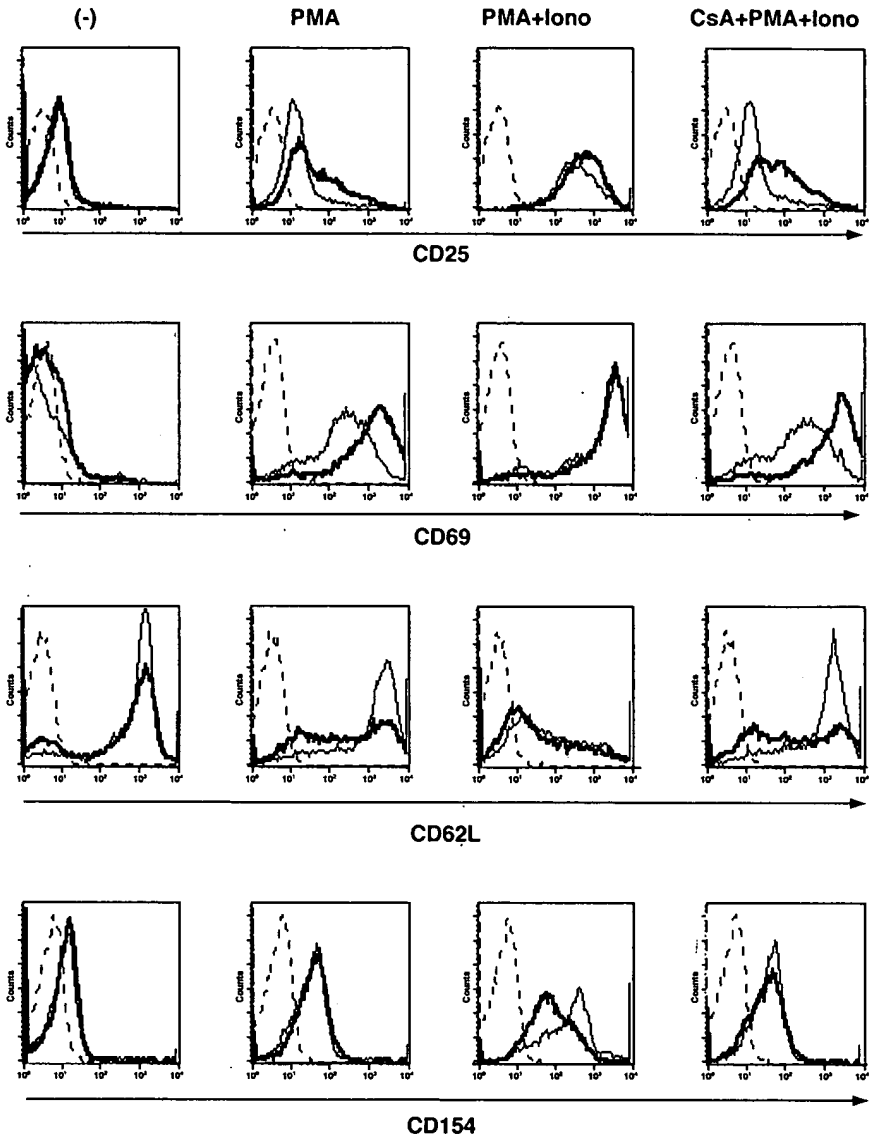

FIGURE 4. Expression patterns of activation/memory T cell surface markers. The expression patterns of the T cell surface markers, CD25, CD69, CD62L, and CD154, were analyzed with flow cytometry 24 h after stimulation with PMA, PMA plus ionomycin, or PMA plus ionomycin plus CsA. Thick lines represent cells from cnNFATx-transgenic mice, thin lines represent wild-type littermates, and dotted lines show background staining with isotype control Ab. Both CD25 and CD69 were up-regulated by the *cnNFATx* transgene. The $CD62L^{high}$ population was reduced in cnNFATx-transgenic mice. Down-regulation of CD62L was also enhanced on $CD4^+$ T cells derived from the cnNFATx-transgenic mice. The enhanced expression of the CD154 by PMA plus ionomycin stimulation was partially suppressed on $CD4^+$ T cells derived from the cnNFATx-transgenic mice.

contribution of NFATx in the IFN-γ expression of the naive T cells is very small.

Regulation of immunoregulatory cell surface molecules by NFATx

In addition to CD25, expression of CD69 and CD62L—known activation markers—was analyzed by flow cytometry (Fig. 4). Similar to CD25, CD69 was partially induced in wild-type T cells treated with PMA and more fully induced in the transgenic T cells. Furthermore, expression of CD69 in transgenic T cells treated with PMA plus ionomycin was resistant to CsA treatment. These data suggest the involvement of NFATx in CD69 expression.

Naive $CD4^+$ T cells express high levels of CD62L, which is suppressed upon T cell activation. Memory cells maintain a low level of CD62L, making this marker a useful tool to distinguish between naive and memory T cells. The number of transgenic $CD4^+$ T cells expressing low levels of CD62L upon PMA stimulation was much higher than that of wild-type T cells, indicating that NFATx down-regulate CD62L expression. The number of naive $CD4^+$ T cells expressing high levels of CD62L from cnNFATx-transgenic mice was reduced compared with wild-type littermates, suggesting a relative expansion of the memory T cell population in cnNFATx-transgenic mice.

CD154 (CD40L) delivers an important signal to B cells and other APC and is required for B cell differentiation (26). It has been reported that the expression of CD154 requires NFAT activity. The expression of CD154 on $CD4^+$ T cells deficient for both NFAT1 and NFATc is severely impaired, indicating that these two NFAT family members are required for the expression of this gene. Interestingly, CD154 expression was reduced on $CD4^+$ T cells derived from cnNFATx-transgenic mice activated by PMA and ionomycin. This may indicate that NFATx inhibits CD154 expression.

Glimcher and colleagues (27) have reported that the expression of the CD95 ligand (CD95L) requires two transcription factors, Egr2 and -3, that are swiftly induced upon T cell activation. Both NFAT1 and NFATx are crucial for the induction of these two transcription factors. However, the expression of CD95L was not affected by the *cnNFATx* transgene. Real-time RT-PCR analysis showed that the expression of Egr family members was not augmented by cnNFATx, suggesting that the transcription of Egr2 and -3 requires additional molecules other than NFAT family members (Fig. 5*a*).

NFATx up-regulates Th1 cytokine genes

Although the expression of IFN-γ from naive $CD4^+$ T cells was affected by cnNFATx, the level of IFN-γ expression from the

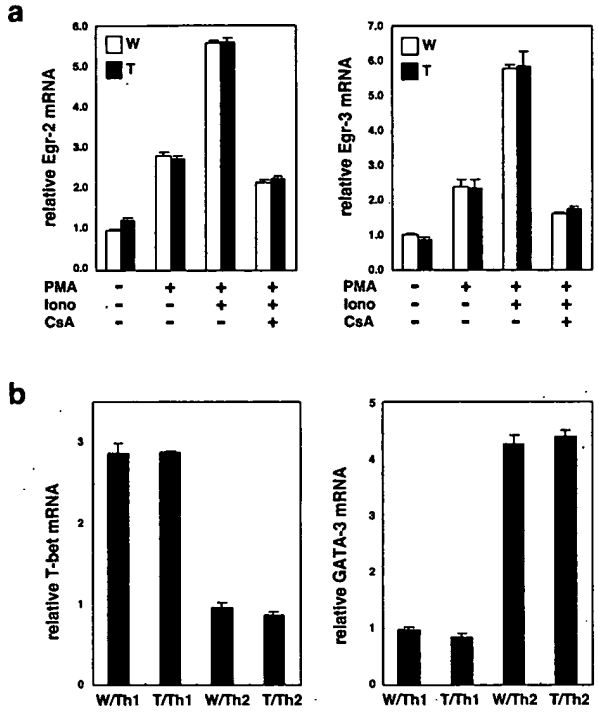

FIGURE 5. Real-time PCR analysis of various transcription factors in differentiated T cells derived from cnNFATx-transgenic mice and wild-type littermates. *a*, mRNA levels of Egr2 and Egr3 in naive T cells are comparable between cnNFATx-transgenic mice and wild-type littermates. $CD4^+CD44^{low}$ naive T cells derived from cnNFATx-transgenic mice (T) and wild-type littermates (W) were stimulated for 4 h with PMA, PMA plus ionomycin, or PMA plus ionomycin plus CsA, and total RNA was isolated. The mRNA level was analyzed by real-time PCR analysis, and the results are normalized to β-actin mRNA. *b*, mRNA levels of T-bet and GATA-3 in in vitro-differentiated Th1 and Th2 are comparable between cnNFATx-transgenic mice and wild-type littermates. $CD4^+CD44^{low}$ naive T cells derived from cnNFATx-transgenic mice (T) and wild-type littermates (W) were stimulated by Ag (OVA peptide) and APC and differentiated under polarizing conditions in vitro for 2 wk. Cells (1×10^7) were then stimulated for 4 h with PMA plus ionomycin, and total RNA was isolated. The real-time PCR analysis of T-bet and GATA-3 mRNA was performed as in *a*.

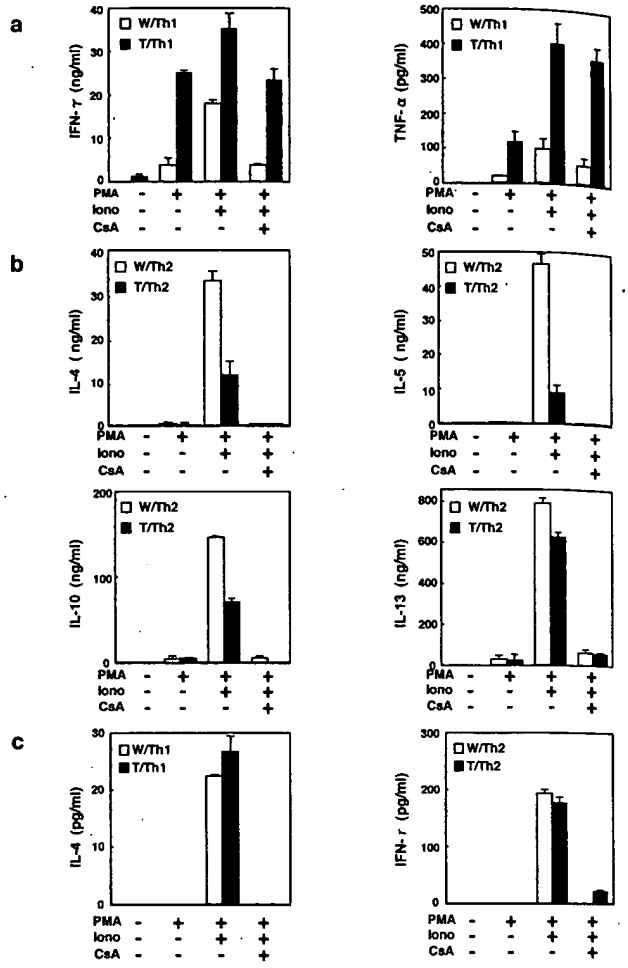

FIGURE 6. Cytokine profile of Th1 or Th2 cells derived from cnNFATx-transgenic mice and wild-type littermates. $CD4^+CD44^{low}$ naive T cells derived from cnNFATx-transgenic mice (T) and wild-type littermates (W) were stimulated by Ag (OVA peptide) and APC and differentiated under polarizing conditions in vitro for 2 wk. Cells (2×10^6) were then stimulated for 24 h with PMA, PMA plus ionomycin, or PMA plus ionomycin plus CsA. Cytokine levels in the supernatants were measured by ELISA. Data from one representative experiment are shown. Th1 cytokines IFN-γ and TNF-α were up-regulated in transgenic Th1 cells (*a*), while Th2 cytokines IL-4, IL-5, IL-10, and IL-13 were suppressed in transgenic Th2 cells (*b*). The expression level of IFN-γ in Th2 cells and IL-4 in Th1 cells was comparable between cells derived from cnNFATx-transgenic mice and wild-type littermates (*c*). *a* and *c* (*left*), ■, T/Th1, Th1 cells derived from cnNFATx-transgenic mice; □, W/Th1, Th1 cells from wild-type littermates. *b* and *c* (*right*), ■, T/Th2, Th2 cells derived from cnNFATx-transgenic mice; □, W/Th2, Th2 cells from wild-type littermates.

naive cells was very low. To analyze the effect of cnNFATx on Th subset-specific cytokine genes, naive $CD4^+$ T cells isolated from either cnNFATx-transgenic mice or wild-type littermates were differentiated in vitro. Th1 or Th2 cells were stimulated with PMA alone, PMA plus ionomycin, or PMA plus ionomycin plus CsA for 24 h, and the amount of various cytokines in the supernatant were analyzed by ELISA (Fig. 6). Th1 cells from the transgenic mice produced dramatically enhanced levels of both IFN-γ and TNF-α upon PMA alone or PMA plus ionomycin treatment (Fig. 6*a*). In contrast, low, comparable levels of these cytokines were produced by Th2 cells derived from the cnNFATx-transgenic mice and the wild-type littermates after PMA plus ionomycin treatment (Fig. 6*c*). The expression level of the transcription factor T-bet, analyzed by the real-time PCR, was not enhanced in T cells derived from cnNFATx-transgenic mice (Fig. 5*b*). These support the idea that cnNFATx does not up-regulate the expression of Th1 cytokine genes by skewing toward Th1.

To determine whether this phenotype is derived from the altered thymocyte selection in the transgenic mice, cnNFATx was introduced into wild-type CD4-positive naive T cells by retrovirus vector bicistronically expressing EGFP. One week after the infection and incubation under the Th1 skewing condition, EGFP-positive and -negative populations were separated by FACS and stimulated again to further differentiate and proliferate under Th1-skewing condition. As shown in Fig. 7, the number of cells expressing IFN-γ is up-regulated with the cnNFATx-transduced population, suggesting that this effect on cytokine genes is not due to the aberrant selection process in the thymus of the transgenic mice.

NFATx down-regulated Th2 cytokine genes

Despite the presence of the *cnNFATx* transgene, Th2 cytokine genes were not induced by treatment with PMA alone, and CsA completely shut off their expression. When Th2 cells were stimulated by PMA and ionomycin, expression of the Th2 cytokine genes *IL-4*, *IL-5*, and *IL-10* was impaired. The level of IL-13 was also reduced, although less effectively, in Th2 cells derived from

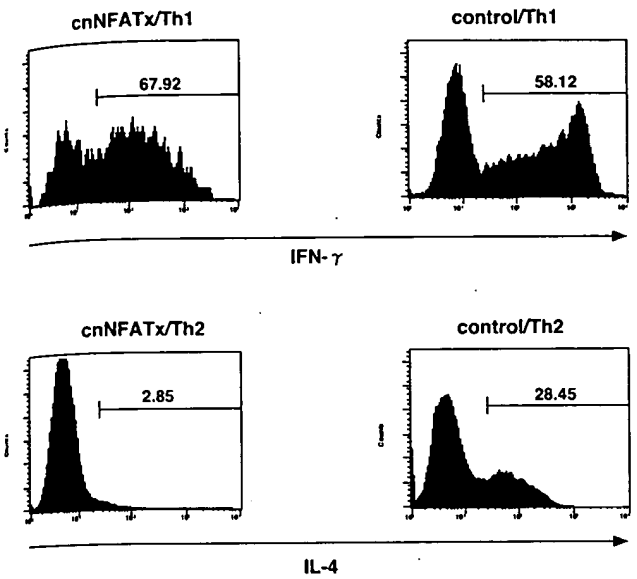

FIGURE 7. Effect of ectopic expression of cnNFATx on the expression of Th1 cytokine IFN-γ and Th2 cytokine IL-4. $CD4^+CD44^{low}$ naive T cells derived from DO11.10 αβ TCR-transgenic mice were stimulated by Ag (OVA peptide) and APC and differentiated under polarizing conditions. On days 2 and 3, cells were transduced with cnNFATx retrovirus. On day 7, EGFP-positive and -negative cells were separated and stimulated again for further differentiation and proliferation. On day 14, cells were stimulated with medium containing PMA and ionomycin and analyzed for the expression of IFN-γ and IL-4 by the intracellular cytokine staining. Data are presented as single-color histograms. The percentage of cells staining positive for each cytokine is indicated. Data are representative of two independent experiments.

cnNFATx-transgenic mice (Fig. 6*b*). A small amount of the Th2 cytokine IL-4 was produced by Th1 cells, and there was no difference between the Th1 cells derived from the cnNFATx-transgenic mice and the wild-type littermates (Fig. 6*c*). The real-time PCR analysis again revealed that the level of GATA3, critical regulator of Th2 differentiation, was not affected by the cnNFATx (Fig. 5*b*). Thus, the effect of the ectopic expression of the cnNFATx is on the individual cytokine genes rather than on the differentiation pathway. In addition, the idea that this phenotype of the transgenic T cells is derived from the altered thymic selection was eliminated by the fact that retrovirally transduced cnNFATx can also suppress IL-4 production in Th2 (Fig. 7).

Discussion

Although $CD4^+$ T cells derived from NFATx-deficient mice show few alterations in the pattern of expression of immunoregulatory genes (15), our findings using cnNFATx-transgenic T cells demonstrate that NFATx is in fact a potent regulatory transcription factor for various cytokines and cell surface molecules. NFATx is a positive regulator for the Th1 cytokine genes *IFN-γ* and *TNF-α* in Th1 cells as well as some activation marker molecules in naive T cells. It has to be pointed out that the effect of cnNFATx on IFN-γ was clearly observed in Th1-differentiated cells, while the effect observed in naive T cells was very subtle. This indicates that NFATx is involved in the regulation of IFN-γ in Th1 effector cells, although it has little effect in naive T cells. NFATx negatively regulates the Th2 cytokine genes *IL-4*, *IL-5*, *IL-13*, and *IL-10*, as well as the important immunoregulatory molecule CD154 (CD40L). Gene disruption experiments have revealed that, although NFAT1 is critical for the induction of *IL-4* gene, it also suppresses the persistent expression of this gene (8–12). Thus, the effect of NFAT1 on the overall production of IL-4 may depend on a subtle balance of various conditions. NFAT1 KO mice become more susceptible to the *Leishmania major* infection than wild-type littermates, suggesting the biased response toward Th2 phenotype probably due to the enhanced production of IL-4 (12). Our findings of the cnNFATx-transgenic mice imply that NFATx is an inhibitor for Th2 cytokine production. In contrast, NFATx positively regulates the expression of the Th1 cytokines. Mice deficient in NFAT1 show reduced level of Th1 signature cytokine IFN-γ, suggesting that NFAT1 is also a positive factor for Th1 cytokines (8–12, 14). Unlike NFATc, both NFAT1 and NFATx are expressed in resting T cells, although the level of NFATx is lower than that of NFAT1 (20, 28). Taken together, these data indicate that NFATx and NFAT1 share similar and overlapping functions in $CD4^+$ T cells.

In contrast to naive $CD4^+$ T cells from wild-type littermates, which do not proliferate in response to the treatment with PMA alone, those derived from the transgenic mice proliferate, albeit slowly, in the presence of PMA. This proliferation results from the production of IL-2 and/or the up-regulation of CD25, as demonstrated by the inhibition of proliferation by mAb against each chain of IL-2R complex (data not shown). These data indicate that NFATx can be a positive regulator for the expression of IL-2, although its contribution may be relatively small due to its small quantity in resting $CD4^+$ T cells. Proliferation of resting T cells in response to Ag binding depends on the TCR signal itself rather than the supply of IL-2 provided by autocrine or paracrine mechanisms. This is evidenced by the fact that, although $CD4^+$ T cells derived from double NFAT1/NFATc KO mice produce only residual amounts of IL-2, they are still able to proliferate in response to a TCR signal (7). Analysis of naive $CD4^+$ T cells derived from double NFAT1/NFATx KO mice has demonstrated that both NFAT1 and NFATx can inhibit proliferation. This is further supported by the observation that the proliferation of double NFAT1/NFATx KO $CD4^+$ T cells shows less dependency on the CD28 costimulatory signal and the shortening of time to first cell cycle division (17). We failed to detect any effect of overexpression of cnNFATx on the proliferation of cells stimulated by PMA and Ca ionophore, perhaps because inhibition of T cell proliferation under these conditions requires both NFAT1 and NFATx.

In transgenic Th1 cells, both IFN-γ and TNF-α (Th1 cytokines) were up-regulated significantly, not only by PMA stimulation but also by full activation with PMA and ionomycin. This observation implies that NFATx can function as a strong positive regulator for these Th1 cytokine genes in differentiated Th1 cells. However, in naive $CD4^+$ T cells, NFATx has little effect on the expression of IFN-γ. This is consistent with the fact that, in the absence of NFATc and NFAT1, the efficient expression of IFN-γ in naive $CD4^+$ T cells is severely impaired (7).

The production of IL-4, IL-5, IL-13, and IL-10 were all reduced significantly in Th2 cells derived from the cnNFATx-transgenic mice, suggesting that NFATx is a negative regulator of these Th2 cytokine genes. NFAT1-deficient T cells exhibit prolonged IL-4 production, which causes skewing toward Th2 development and leads to allergic inflammation. Th2 cells predominate in double NFAT1/NFATx KO mice, which suffer from lymphoproliferative disorders, such as allergic pneumonitis and blepharitis, associated with increases in serum levels of IgE and IgG1 (16). Because overproduction of IL-4 has been shown in transgenic mice to inhibit T cell proliferation (29), the Th2-dominant lymphoproliferative state of the double NFAT1/NFATx KO mice is presumably due to the relief of the inhibitory effect of NFAT1 and NFATx on both the proliferation of $CD4^+$ T cells and the expression of Th2 cytokines such as IL-4 to stimulate Th2 differentiation in vivo.

It could be argued that the up-regulation of Th1 cytokines and the down-regulation of Th2 cytokines in response to the overproduction of cnNFATx indicate that cnNFATx facilitates Th1 differentiation. However, Th2 cells from cnNFATx-transgenic mice produce levels of IFN-γ that are comparable to those of wild-type littermates (Fig. 6*c*), and Th1 cells from these transgenic mice produce about the same, extremely low levels of IL-4 as do their wild-type littermates. In addition, real-time RT-PCR analysis revealed that both transgenic and wild-type Th1 and Th2 cells have comparable levels of the Th1- and Th2-specific master regulatory genes, *T-bet* and *GATA3*, respectively (Fig. 5*b*) (30–34). Therefore, NFATx affects individual cytokine genes that are selected for the particular lineage rather than affecting the overall differentiation process.

Another argument is that the effect of NFATx on the expression of those cytokine genes is due to the selection of particular type of T cells in the thymus of transgenic mice, because cnNFATx is strongly driven in the thymus by the *lck* proximal promoter. However, similar effects of cnNFATx on the expression of cytokine genes were observed by CD4+ naive T cells expressing retrovirally transduced cnNFATx (Fig. 7), indicating that the effects of cnNFATx are not the results of thymic selection. The effect of the transgene on Th1 cytokine genes was more prominent and clearly detected than that on Th2 cytokine genes at an earlier time point (1 wk after differentiation, data not shown) in T cells derived from cnNFATx-transgenic mice. On the contrary, the effect of the retrovirally transduced cnNFATx was more remarkable on Th2 cytokine genes. Because gene transduction by a retrovirus vector requires activation and proliferation of naive T cells, one possible interpretation for the difference between these two experimental systems may be the effect of the cnNFATx protein in the resting naive T cells derived from the transgenic mice.

A number of immunoregulatory molecules expressed on the cell surface are also differentially regulated by NFATx. Activation markers such as CD69 and CD25 are up-regulated by the *cnNFATx* transgene. CD25 is induced by TCR signal and its high expression level is maintained partly by the IL-2-STAT5a/STAT5b signaling pathway (35–37). The up-regulation of CD25 by PMA stimulation in transgenic T cells is probably due to a direct effect of NFATx on the CD25 promoter rather than to the up-regulation of IL-2, which induces CD25 only late in activation. The expression of CD154 (CD40L) was negatively regulated by cnNFATx, in contrast to NFAT1 and NFATc, which are both required for the induction of CD154 (CD40L) in naive CD4+ T cells (7). This suppressive effect of NFATx on CD154 (CD40L), which may be unique among NFAT family members expressed in T cells, was also observed when transgenic naive CD4+ T cells were stimulated solely by anti-CD3 Ab or by both anti-CD3 and anti-CD28 Abs (data not shown). CD154 (CD40L) expression is crucial for the activation and differentiation of B cells and other APC (26), and CD154 (CD40L) signaling can further activate CD4+ T cells. Thereby, in addition to suppressing the proliferation of naive CD4+ T cells and the expression of Th2 cytokines, NFATx may also maintain the homeostasis of the immune response by suppressing CD154 (CD40L) expression. A gene disruption experiment has shown that NFAT1 and NFATx regulate the expression of CD95L through the transcription factors Egr2 and -3 (27). Because the *NFATx* transgene alone up-regulated neither CD95L nor Egr2/3 expression, NFAT1 and NFATx are not sufficient for the regulation of CD95L through the induction of Egr2/3 (Fig. 5 and data not shown).

Clipstone and colleague (38) have reported the effects of the retrovirally transduced, constitutively active form of NFATc on various immunoregulatory genes including cytokines in CD4+ T cells. They concluded that the constitutively active form of NFATc commits cells to the Th1 phenotype, resulting in the up-regulation of Th1-specific genes, including *IFN-γ*, and the down-regulation of Th2-specific genes such as *IL-4*. They also detected numbers of Th0 cells producing both IFN-γ and IL-4. These observations imply that a retrovirally transduced, constitutively active form of NFATc phenocopies the strong and repetitive stimuli through TCR that favor Th1 differentiation. They speculate that this effect is a characteristic of all constitutively active forms of NFAT family members rather than a unique feature of NFATc.

We believe our observations can be attributed to unique features of NFATx rather than to a general effect common to all constitutively active NFAT family members. First, the effect of the *cnNFATx* transgene was limited to cytokines that were specific to the particular lineage being studied. Although cnNFATx down-regulates four Th2 cytokine genes and up-regulates two Th1 cytokine genes, it did not affect the expression levels of master regulatory genes of Th subset differentiation, *GATA3* and *T-bet*, suggesting that NFATx is not changing the polarity of Th subset differentiation. One possible mechanism is the competition between NFATx and other NFAT family members for the promoter of those cytokine genes. NFATx may be a less potent activator for those Th2 cytokine gene promoters while more potent for those Th1 cytokine promoters. Second, exogenous NFATc seems to have a very strong effect, indicated by the appearance of Th0-type cells. In contrast, ectopic NFATx expression derived from either the transgene or retrovirally transduced gene did not induce Th0-type cells, suggesting that NFATx is not as strong as NFATc (data not shown). Finally, retrovirally transduced NFATc up-regulates both CD154 (CD40L) and CD95L, whereas the presence of the *NFATx* transgene did not affect the expression of CD95L and down-regulated CD154 (CD40L).

Further studies on the precise functions of each NFAT family member should offer important information on the role of the NFAT family network in T cell activation and the differentiation of effector cells.

Acknowledgments

We thank Drs. Roger M. Perlmutter and Garry P. Nolan for kindly supplying the plasmid carrying *lck* proximal promoter used in making transgenic mice and the packaging cell line (Phoenix-Eco), respectively.

References

1. Kiani, A., A. Rao, and J. Aramburu. 2000. Manipulating immune responses with immunosuppressive agents that target NFAT. *Immunity 12:359.*
2. Crabtree, G. R. 1999. Generic signals and specific outcomes: signaling through Ca^{2+}, calcineurin, and NF-AT. *Cell 96:611.*
3. Rao, A., C. Luo, and P. G. Hogan. 1997. Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol. 15:707.*
4. Crabtree, G. R., and E. N. Olson. 2002. NFAT signaling: choreographing the social lives of cells. *Cell 109:S67.*
5. Ranger, A. M., M. R. Hodge, E. M. Gravallese, M. Oukka, L. Davidson, F. W. Alt, F. C. de la Brousse, T. Hoey, M. Grusby, and L. H. Glimcher. 1998. Delayed lymphoid repopulation with defects in IL-4-driven responses produced by inactivation of NF-ATc. *Immunity 8:125.*
6. Yoshida, H., H. Nishina, H. Takimoto, L. E. Marengere, A. C. Wakeham. D. Bouchard, Y. Y. Kong, T. Ohteki, A. Shahinian, M. Bachmann, et al. 1998. The transcription factor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. *Immunity 8:115.*
7. Peng, S. L., A. J. Gerth, A. M. Ranger, and L. H. Glimcher. 2001. NFATc1 and NFATc2 together control both T and B cell activation and differentiation. *Immunity 14:13.*
8. Schuh, K., B. Kneitz, J. Heyer, F. Siebelt, C. Fischer, E. Jankevics, E. Rude, E. Schmitt, A. Schimpl, and E. Serfling. 1997. NF-ATp plays a prominent role in the transcriptional induction of Th2-type lymphokines. *Immunol. Lett. 57:171.*
9. Schuh, K., B. Kneitz, J. Heyer, U. Bommhardt, E. Jankevics, F. Berberich-Siebelt, K. Pfeffer, H. K. Muller-Hermelink, A. Schimpl, and E. Serfling. 1998. Retarded thymic involution and massive germinal center formation in NF-ATp-deficient mice. *Eur. J. Immunol. 28:2456.*
10. Hodge, M. R., A. M. Ranger, F. Charles de la Brousse, T. Hoey, M. J. Grusby, and L. H. Glimcher. 1996. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. *Immunity 4:397.*

11. Xanthoudakis, S., J. P. Viola, K. T. Shaw, C. Luo, J. D. Wallace, P. T. Bozza, D. C. Luk, T. Curran, and A. Rao. 1996. An enhanced immune response in mice lacking the transcription factor NFAT1. *Science 272:892.*
12. Kiani, A., J. P. Viola, A. H. Lichtman, and A. Rao. 1997. Down-regulation of *IL-4* gene transcription and control of Th2 cell differentiation by a mechanism involving NFAT1. *Immunity 7:849.*
13. Viola, J. P., A. Kiani, P. T. Bozza, and A. Rao. 1998. Regulation of allergic inflammation and eosinophil recruitment in mice lacking the transcription factor NFAT1: role of interleukin-4 (IL-4) and IL-5. *Blood 91:2223.*
14. Kiani, A., F. J. Garcia-Cozar, I. Habermann, S. Laforsch, T. Aebischer, G. Ehninger, and A. Rao. 2001. Regulation of interferon-γ gene expression by nuclear factor of activated T cells. *Blood 98:1480.*
15. Oukka, M., I. C. Ho, F. C. de la Brousse, T. Hoey, M. J. Grusby, and L. H. Glimcher. 1998. The transcription factor NFAT4 is involved in the generation and survival of T cells. *Immunity 9:295.*
16. Ranger, A. M., M. Oukka, J. Rengarajan, and L. H. Glimcher. 1998. Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. *Immunity 9:627.*
17. Rengarajan, J., B. Tang, and L. H. Glimcher. 2002. NFATc2 and NFATc3 regulate Th2 differentiation and modulate TCR-responsiveness of naive Th cells. *Nat. Immunol. 3:48.*
18. Amasaki, Y., S. Adachi, Y. Ishida, M. Iwata, N. Arai, K. I. Arai, and S. Miyatake. 2002. Constitutively nuclear form of NFATx shows efficient transactivation activity and induces differentiation of $CD4^+CD8^+$ T cells. *J. Biol. Chem. 7:7.*
19. Adachi, S., Y. Amasaki, S. Miyatake, N. Arai, and M. Iwata. 2000. Successive expression and activation of NFAT family members during thymocyte differentiation. *J. Biol. Chem. 275:14708.*
20. Garvin, A. M., S. Pawar, J. D. Marth, and R. M. Perlmutter. 1988. Structure of the murine *lck* gene and its rearrangement in a murine lymphoma cell line. *Mol. Cell. Biol. 8:3058.*
21. Murphy, E., K. Shibuya, N. Hosken, P. Openshaw, V. Maino, K. Davis, K. Murphy, and A. O'Garra. 1996. Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. *J. Exp. Med. 183:901.*
22. Amasaki, Y., E. S. Masuda, R. Imamura, K. Arai, and N. Arai. 1998. Distinct NFAT family proteins are involved in the nuclear NFAT-DNA binding complexes from human thymocyte subsets. *J. Immunol. 160:2324.*
23. Kinsella, T. M., and G. P. Nolan. 1996. Episomal vectors rapidly and stably produce high-titer recombination retrovirus. *Hum. Gene Ther. 7:1405.*
24. Ferber, R., H. J. Lee, F. Zonin, V. Heath, A. Mui, N. Arai, and A. O'Garra. 1999. GATA-3 significantly downregulates IFN-γ production from developing Th1 cells in addition to inducing IL-4 and IL-5 levels. *Clin. Immunol. 91:134.*
25. Kurata, H., H. J. Lee, A. O'Garra, and N. Arai. 1999. Ectopic expression of activated Stat6 induces the expression of Th2-specific cytokines and transcription factors in developing Th1 cells. *Immunity 11:677.*
26. Grewal, I. S., and R. A. Flavell. 1996. The role of CD40 ligand in costimulation and T-cell activation. *Immunol. Rev. 153:85.*
27. Rengarajan, J., P. R. Mittelstadt, H. W. Mages, A. J. Gerth, R. A. Kroczek, J. D. Ashwell, and L. H. Glimcher. 2000. Sequential involvement of NFAT and Egr transcription factors in FasL regulation. *Immunity 12:293.*
28. Lyakh, L., P. Ghosh, and N. R. Rice. 1997. Expression of NFAT-family proteins in normal human T cells. *Mol. Cell. Biol. 17:2475.*
29. Tepper, R. I., D. A. Levinson, B. Z. Stanger, J. Campos-Torres, A. K. Abbas, and P. Leder. 1990. IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice. *Cell 62:457.*
30. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell 89:587.*
31. Ho, I. C., and L. H. Glimcher. 2002. Transcription: tantalizing times for T cells. *Cell 109:S109.*
32. Glimcher, L. H., and K. M. Murphy. 2000. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev. 14:1693.*
33. Murphy, K. M., W. Ouyang, J. D. Farrar, J. Yang, S. Ranganath, H. Asnagli, M. Afkarian, and T. L. Murphy. 2000. Signaling and transcription in T helper development. *Annu. Rev. Immunol. 18:451.*
34. Glimcher, L. H., and H. Singh. 1999. Transcription factors in lymphocyte development: T and B cells get together. *Cell 96:13.*
35. Waldmann, T. A. 1989. The multi-subunit interleukin-2 receptor. *Annu. Rev. Biochem. 58:875.*
36. Nakajima, H., X. W. Liu, A. Wynshaw-Boris, L. A. Rosenthal, K. Imada, D. S. Finbloom, L. Hennighausen, and W. J. Leonard. 1997. An indirect effect of Stat5a in IL-2-induced proliferation: a critical role for Stat5a in IL-2-mediated IL-2 receptor α chain induction. *Immunity 7:691.*
37. Schuh, K., T. Twardzik, B. Kneitz, J. Heyer, A. Schimpl, and E. Serfling. 1998. The interleukin 2 receptor α chain/CD25 promoter is a target for nuclear factor of activated T cells. *J. Exp. Med. 188:1369.*
38. Porter, C. M., and N. A. Clipstone. 2002. Sustained NFAT signaling promotes a Th1-like pattern of gene expression in primary murine $CD4^+$ T cells. *J. Immunol. 168:4936.*